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Citation for published version:

Hasenpusch-Theil, K, Watson, JA & Theil, T 2015, 'Direct interactions between Gli3, Wnt8b and Fgfs underlie patterning of the dorsal telencephalon', *Cerebral Cortex*. <https://doi.org/10.1093/cercor/bhv291>

Digital Object Identifier (DOI):

[10.1093/cercor/bhv291](https://doi.org/10.1093/cercor/bhv291)

Link:

[Link to publication record in Edinburgh Research Explorer](#)

Document Version:

Peer reviewed version

Published In:

Cerebral Cortex

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Direct interactions between *Gli3*, *Wnt8b* and *Fgfs* underlie patterning of the dorsal telencephalon

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Key words: *Fgf15*, *Fgf17*, *Gli3*, patterning, *Wnt8b*

ABSTRACT

A key step in the development of the cerebral cortex is a patterning process which subdivides the telencephalon into several molecularly distinct domains and is critical for cortical arealisation. This process is dependent on a complex network of interactions between signalling molecules of the *Fgf* and *Wnt* gene families and the *Gli3* transcription factor gene but a better knowledge of the molecular basis of the interplay between these factors is required to gain a deeper understanding of the genetic circuitry underlying telencephalic patterning. Using DNA binding and reporter gene assays, we here investigate the possibility that *Gli3* and these signalling molecules interact by directly regulating each other's expression. We show that Fgf signalling is required for *Wnt8b* enhancer activity in the cortical hem while Wnt/ β -catenin signalling represses *Fgf17* forebrain enhancer activity. In contrast, Fgf and Wnt/ β -catenin signalling cooperate to regulate *Gli3* expression. Taken together, these findings indicate that mutual interactions between *Gli3*, *Wnt8b* and *Fgf17* are crucial elements of the balance between these factors thereby conferring robustness to the patterning process. Hence, our study provides a framework for understanding the genetic circuitry underlying telencephalic patterning and how defects in this process can affect the formation of cortical areas.

INTRODUCTION

A patterning process subdividing the telencephalon into several distinct domains represents a key step in the development of the cerebral cortex which performs highly complex neural tasks. This process is thought to be critically dependent on a complex network of interactions between intercellular signalling molecules and transcription factors. Signalling molecules of the *Fgf*, *Wnt* and *Bmp* gene families act as morphogens to determine rostradorsal and dorsomedial telencephalic cell fates (Malumbres M et al. 2004; Machon O et al. 2007; Mangale VS et al. 2008), respectively, by directly controlling the expression of transcription factors (Theil T et al. 2002; Lai T et al. 2008). In turn, these factors regulate not only the expression of genes involved in cell fate determination and in controlling proliferation/differentiation of cortical progenitor cells but also that of the signalling molecules themselves thereby setting up feedback loops between signalling molecules and transcription factors (Del Rio JA et al. 2000; Theil T et al. 2002; Saulnier A et al. 2013). Moreover, the signalling molecules have been shown to auto-regulate their own expression (Hasenpusch-Theil K et al. 2012) or to repress each other's expression (Ohkubo Y et al. 2002; Shimogori T et al. 2004; Storm EE et al. 2006). Collectively, these complex interactions are used to establish and to maintain distinct gene expression territories in the developing telencephalon as a prerequisite for patterning.

Interactions between *Fgfs* and *Bmps/Wnts* are a crucial element in the model outlined above. Analyses of mutant mice and studies in chicken embryos have revealed that the disruption of a tight balance between *Fgfs* and *Bmp/Wnts* leads to severe defects in dorsal telencephalic development (Crossley PH et al. 2001; Ohkubo Y et al. 2002; Shimogori T et al. 2004; Storm EE et al. 2006). A key factor in regulating this balance is the Gli3 zinc finger transcription factor. In the dorsal telencephalon, the Gli3 repressor form predominates (Fotaki V et al. 2006) to inhibit Sonic hedgehog (Shh) signalling but it also represses the expression of multiple Fgf family members (*Fgf8/15/17/18*) and is required for the expression of *Bmp2/4/6* and *Wnt3a/7b/8b* (Grove EA et al. 1998; Theil T et al. 1999; Kuschel S et al. 2003; Rash BG and EA Grove 2007). Absence or reduced levels of *Gli3* result in the up-regulation of these *Fgfs* and in a down-regulation of *Bmp/Wnt* gene expression. These changes severely affect telencephalic patterning (Grove EA et al. 1998; Theil T et al. 1999; Tole S et al. 2000; Kuschel S et al. 2003) and also later processes in cortical development such as the formation of the corpus callosum (Vokes SA et al. 2008; Speksnijder L et al. 2013; Magnani D et

al. 2014). These findings therefore emphasize the importance of a tightly controlled balance between *Gli3*, *Fgfs* and *Bmps/Wnts* for normal cortical development. However, these interactions are mainly derived from genetic analyses and very little is known about their molecular basis. A better knowledge of the interactions at the molecular level is required for a deeper understanding of the molecular and genetic circuitry underlying telencephalic patterning.

Here, we explore the possibility that *Wnts*, *Fgfs* and *Gli3* regulate each other's expression through direct transcriptional regulation. Using DNA binding, reporter gene assays and overexpression experiments we show that a *Wnt8b* dorsomedial telencephalon enhancer contains an essential binding site for Ets transcription factors, transcriptional mediators of Fgf signalling. Mutation of this site results in loss of *lacZ* reporter gene expression in the cortical hem. In turn, a binding site for Tcf transcription factors is required to repress the activity of an *Fgf17* forebrain enhancer in the dorsomedial telencephalon. Moreover, we show that the activity of a *Gli3* dorsal telencephalon enhancer depends on Fgf signalling. Mutations of an Ets binding site within this enhancer abolish its activity in the dorsomedial telencephalon. Interestingly, this Ets site flanks a Tcf binding site which we previously showed to be essential for *Gli3* enhancer activity (Hasenpusch-Theil K *et al.* 2012). Indeed, the presence of Ets protein increases the binding of Lef1 to the *Gli3* enhancer in electrophoretic mobility shift assays (EMSAs). Taken together, these findings suggest complex and direct interactions between *Gli3*, *Wnt8b* and *Fgf17* including direct (i) mutual interactions between Wnt and Fgf signalling and (ii) a cooperative interaction between these signalling pathways to regulate *Gli3* expression.

MATERIAL AND METHODS

Mice

Gli3^{Xt/+} and *Gli3*^{Pdn/+} animals were kept on a mixed C57Bl6/C3H and C3H background, respectively, and were interbred. Embryonic (E) day 0.5 was assumed to start at midday of the day of vaginal plug discovery. Embryos were genotyped as described (Maynard TM et al. 2002; Ueta E et al. 2002). For in situ hybridisation, *Gli3*^{Xt/+}, *Gli3*^{Pdn/+} and wild-type embryos which did not show differences were used as control embryos and forebrain morphology was used to distinguish them from *Gli3*^{Xt/Pdn} and *Gli3*^{Pdn/Pdn} embryos (Kuschel S et al. 2003). *Emx1Cre* and *Gli3*^{flox/flox} mouse lines have been described previously (Gorski JA et al. 2002; Blaess S et al. 2008). For *Emx1Cre;Gli3*^{flox/flox} conditional embryos, *Gli3*^{flox/flox}, *Gli3*^{flox/+}, *Emx1Cre* and *Gli3*^{flox/+} embryos were used as controls. All experimental procedures involving mice were performed in accordance with local guidelines. For each marker and each stage, 3-5 embryos were analysed.

In situ hybridisation, immunohistochemistry and X-Gal staining on sectioned embryonic brains

In situ hybridisation on 10 µm coronal paraffin sections of E12.5 mouse brains were performed as described previously (Theil T 2005). Digoxigenin-labeled antisense probes were generated from the following cDNA clones: *Fgf15* (Lopez-Rios J et al. 2012), *Fgf17* (Fry DW et al. 2004), *Fgf18* (Finn RS et al. 2009).

For the reporter gene analysis of in utero electroporated embryos, brains were dissected in PBS and fixed for 3 hours in 4% PFA. After embedding in OCT/sucrose, 14 µm coronal cryosections were analysed by immunofluorescence using an antibody against GFP (1:1000; Abcam), followed by a nuclear counterstain with TO-PRO-1 (1:3000, Invitrogen) as described previously (Hasenpusch-Theil K et al. 2012). Adjacent sections were stained o/n with X-Gal at 37°C and counterstained with Fast RED (Hasenpusch-Theil K et al. 2012).

Plasmid construction and mutagenesis

All genomic DNA fragments were generated via PCR using wild-type genomic DNA (for oligonucleotides see Supplementary Tab. 1). Enhancer sequences were subcloned using a TOPO TA cloning kit (Invitrogen) and verified by sequencing. Putative TCF/Lef1 and Ets binding sites were mutated using the QuickChange Site-Directed Mutagenesis Kit (Stratagene) (for oligonucleotides used in mutagenesis see Supplementary Tab. 2). All mutations were confirmed by sequencing. To test for enhancer activity, wild-type and mutant regulatory elements were subcloned into the *lacZ* reporter gene vector pGZ40 upstream of a human β -globin minimal promoter (Yee SP and PW Rigby 1993). For generating transgenic embryos, the enhancer/reporter fragment was released from the plasmid backbone by digestion with the restriction enzymes indicated in Supplementary Tab. 1 and gel purified.

For the Etv4 activator and repressor constructs, the Etv4 DNA binding domain was PCR amplified using the forward oligonucleotide 5'CAGCGTCGTGGCTCACTTC3' and the reverse oligonucleotide 5'AATACTAGTAAAGCTTCTGGCTCACACAC3'. The resulting PCR fragment was subcloned into pCS2+ vector upstream and in frame of either the Engrailed repressor domain or the E1A activator domain (Bellefroid EJ et al. 1996).

Electrophoretic mobility shift assay

Electrophoretic mobility assays for the *Wnt8b* and *Gli3* enhancers were performed with radioactively labelled oligonucleotides using purified GST, GST-Etv4 (Pollen AA et al. 2014) and GST-Lef1 as described previously (Theil T et al. 2002). For the *Fgf17* enhancers, biotin labelled oligonucleotides were used for bandshift analysis. The binding reaction were separated on native 5% acrylamide gels and transferred onto positively charged nylon membranes (Roche) with a Perfect Blue Semi-dry electro blotter (60 minutes at 120volts, 5mA). After UV crosslinking, biotin labelled probes were detected using a Chemiluminiscent Nucleic Acid Detection Module (Thermo Scientific #89880) according to manufacturer's instructions and imaged using a Kodak BioMaxXAR film.

For oligonucleotide sequences covering the wild-type or mutated Etv4 and Tcf/Lef binding sites see Supplementary Tab. 3. The exchanged nucleotides in the mutated forms are underlined. Wild-type, Ets and Tcf/Lef binding site mutant oligonucleotides were used as specific and unspecific competitors, respectively, in a 10- to 100-fold molar excess.

Transgenic embryos

Transgenic embryos were generated by microinjection of fertilised eggs from B6CBAF1/Crl crosses (Charles River) and were identified by PCR using extra-embryonic yolk sac or tail DNA. Expression of the transgene was analysed by staining E10.5 or E11.5 embryos for β -galactosidase activity as described previously (Theil T et al. 1998).

In utero electroporation

E12.5 pregnant mice were anesthetized with sodium pentobarbitone at 50 mg per gram of body weight and the uterine horns were exposed. *LacZ* reporter gene plasmids, a *GFP* expression plasmid and *Etv4* repressor/activator plasmids were co-injected into the lateral ventricle at 1mg/ml each with a glass micropipette. The embryo in the uterus was placed between CUY650 tweezer-type electrodes (Nepagene). A CUY21E electroporator (Nepagene) was used to deliver six pulses (30 V, 50 ms each) at intervals of 950 ms. The uterine horns were placed back into the abdominal cavity and embryos were allowed to develop for 24 hours before further processing for immunofluorescence. For each construct and time point, at least 4 different embryos were analyzed.

Explant culture

Organotypic slice cultures of the E13.5 embryonic mouse telencephalon were prepared as previously described (Magnani D et al. 2010). Brain slices were cultured on polycarbonate culture membranes (8 μ m pore size; Corning Costar) in organ tissue dishes containing 1 ml of medium (Neurobasal/B-27 [Gibco] supplemented with glutamine, glucose, penicillin and streptomycin) in the presence of either DMSO or of 25 μ M or 50 μ M CHIR (Cambridge BioScience). Slices were cultured for 24hrs, fixed with 4% PFA and processed for in situ hybridization as described above.

RESULTS

Interactions between *Gli3*, *Fgfs* and *Wnts/Bmps* play an important role in controlling telencephalic patterning. To gain insights into the molecular mechanisms underlying these interactions we examined the transcriptional regulation of *Gli3* and of members of the *Fgf* and *Wnt* gene families. We used sequence analyses of regulatory elements controlling forebrain expression of *Gli3*, *Fgfs* or *Wnt8b* to identify potential binding sites for Ets and Tcf/Lef transcription factors, transcriptional mediators of Fgf and Wnt/ β -catenin signalling, respectively. We then tested the functionality of these binding sites in DNA binding and reporter gene assays.

Fgf signalling controls cortical hem activity of the *Wnt8b* dorsomedial telencephalon enhancer

An ultraconserved enhancer element located between *Wnt8b* and *Sec31b* drives expression of a *lacZ* reporter gene in the dorsomedial telencephalon in a pattern highly similar to that of the *Wnt8b* gene (Hasenpusch-Theil K *et al.* 2012; Visel A *et al.* 2013; Pattabiraman K *et al.* 2014). We recently showed that this element contains an essential binding site for Lef/Tcf transcription factors suggesting that *Wnt8b* autoregulates its own expression (Hasenpusch-Theil K *et al.* 2012). Here, we investigated the possibility that Fgf signalling directly regulates the activity of the *Wnt8b* dorsomedial telencephalon enhancer. Fgf signalling results in the activation of Ets transcription factors which bind to a purine rich 8bp binding motif (^C/_G/_AAGGAA^G/_AT) (Choi YJ and L Anders 2014). Interestingly, the *Wnt8b* enhancer contains an evolutionarily conserved AAGGAAAT/_C motif which closely resembles this consensus sequence. We therefore determined whether an Ets transcription factor can bind to this sequence from the *Wnt8b* enhancer in electromobility shift assays (EMSAs). During cortical development, Etv1, Etv4, and Etv5 which belong to the same subclass of Ets transcription factors with nearly identical DNA binding specificities (Bielas SL *et al.* 2009) are highly expressed in the rostromedial telencephalon (Zimmer C *et al.* 2010). Since the full length Etv proteins contain an inhibitory domain which interferes with DNA binding and since this inhibition is only relieved upon Fgf/Map Kinase signalling we used a previously described Glutathione-S-transferase fusion protein

(GST-Etv4) containing just the Etv4 DNA binding domain but not the inhibitory domain in the DNA binding assay (Pollen AA *et al.* 2014). Incubation of GST-Etv4 with an oligonucleotide containing the Ets motif from the *Wnt8b* enhancer resulted in the formation of a slower migrating complex (Fig. 1A). To further analyze the specificity and affinity of DNA binding, competition assays were conducted in the presence of various surplus unlabelled wild-type oligonucleotide (competitor) which resulted in progressively diminished binding of GST-Etv4 fusion protein with increasing amounts of competitor. However, the formation of this complex was not competed by unlabelled *Wnt8b* competitor oligonucleotide containing an AGG to CTT exchange which abolishes Ets binding (Magnani D *et al.* 2013). Thus, GST-Etv4 specifically bound to the Ets binding site within the *Wnt8b* dorsomedial telencephalon enhancer.

The role of the Ets binding site in regulating the *Wnt8b* enhancer activity *in vivo* was determined by generating the same point mutations used in the EMSA analyses in the Ets binding site within the *Wnt8b* enhancer. Reporter gene constructs containing either the wild-type or Ets mutant enhancers fused to a *lacZ* reporter gene under the control of a human β -globin minimal promoter (Yee SP and PW Rigby 1993) were used to generate transgenic embryos. In E11.5 embryos transgenic for a *Wnt8b* enhancer/*lacZ* transgene X-Gal staining was detected in the cortical hem, in progenitor cells of the dorsomedial telencephalon and in preplate neurons (n=5/7 transgenic embryos) (Fig. 1B, C). Mutations in the Ets binding site resulted in similar reporter gene expression in cortical progenitor cells and in preplate neurons although *lacZ*⁺ neurons were detected at the level of the ventral telencephalon (n=5/7) (Fig. 1G, H). In contrast, *lacZ* staining was abolished in the cortical hem (n=5/7) suggesting that the Ets binding site is specifically required for *Wnt8b* enhancer activity in the hem.

To further explore the possibility that Fgf signalling regulates hem *Wnt8b* expression, we co-electroporated the *Wnt8b* enhancer construct with Etv4 expression constructs in which the Etv4 DNA binding domain was fused either to an engrailed repressor (Etv4-EngR) or an Adenovirus E1A activator domain (Etv4-E1A) into the rostromedial telencephalon. The resulting fusion proteins have the Etv4 DNA binding domain but act as transcriptional repressor and activator of Etv4 target genes, respectively. Electroporation of just the *Wnt8b* enhancer/*lacZ* reporter construct into the rostromedial telencephalon resulted in strong X-Gal staining in electroporated cells in the ventral rostromedial

telencephalon (n=5 electroporated embryos) but not in more dorsal regions despite strong electroporation in this area (Fig. 1D, I). Co-electroporation of the *Etv4*-E1A expression construct resulted in the robust activation of the reporter in a dorsally expanded domain (n=6 electroporated embryos) (Fig. 1E, J). In contrast, we only detected weakly X-Gal⁺ cells confined to the rostroventral telencephalon after co-electroporation of the *Etv4*-EngR construct (n=5 electroporated embryos) despite similar electroporation efficiencies (Fig. 1F, K). Taken together, these data indicate that *Etv* transcription factor can bind to an essential site within the *Wnt8b* enhancer and regulate its activity in the cortical hem.

Expression of several *Fgf* family members is differentially regulated in *Gli3* mutants

We next analysed how the forebrain expression of *Fgf* genes is regulated. Several *Fgfs* including *Fgf8*, *Fgf15*, *Fgf17* and *Fgf18* show overlapping but distinct expression patterns in the rostral telencephalon. To investigate whether their expression might be regulated by *Gli3*, *Bmp* and/or *Wnt*/β-catenin signalling, we first analysed their expression in various *Gli3* mutants in which *Bmp*/*Wnt* gene expression is differentially affected. The *Gli3* compound mutant *Gli3*^{Xt/Pdn} carries the *Gli3* null allele *extra-toes* (*Xt*^J) over the *Gli3* hypomorphic allele *Polydactyly Nagoya* (*Pdn*) and shows severely reduced *Bmp* signalling and a lack of *Wnt*/β-catenin signalling in the dorsomedial telencephalon while the *Gli3* hypomorphic mutant *Gli3*^{Pdn/Pdn} displays reduced activity of the *Bmp* and *Wnt*/β-catenin pathways (Kuschel S *et al.* 2003; Friedrichs M *et al.* 2008; Speksnijder L *et al.* 2013). In contrast, *Emx1Cre*;*Gli3*^{fl/fl} (*Gli3*^{cKO}) embryos in which *Gli3* is specifically inactivated in the dorsal telencephalon show normal *Bmp4* and *Wnt7b/8b* gene expression at the E12.5 corticoseptal boundary, but show reduced *Bmp*/*Wnt* gene expression in the caudal cortical hem (Vokes SA *et al.* 2008; Hasenpusch-Theil K *et al.* 2012). Comparing *Fgf* gene expression patterns in these different *Gli3* mutants therefore provided us with first insights whether *Gli3*, *Bmps* and/or *Wnts* could regulate *Fgf* gene expression.

Fgf8 is the major *Fgf* signalling molecule with crucial roles in telencephalic development (Louvi A and EA Grove 2011; van den Aamele J *et al.* 2014). Its expression is confined to the commissural plate (CP) in E12.5 control embryos and expands caudomedially in *Gli3*^{Xt/Pdn} and

Gli3^{Pdn/Pdn} embryos (Kuschel S *et al.* 2003) but not in *Gli3*^{ckO} embryos (Vokes SA *et al.* 2008) suggesting that *Fgf8* expression is repressed by *Gli3* in a Bmp and/or Wnt/ β -catenin dependent manner. *Fgf18* expression is also detected in the CP but extends further rostrally into the septum with a sharp expression boundary at the corticoseptal boundary (CSB). *Fgf18* expression is not affected in *Gli3* mutants (Sup. Fig. 1) indicating that its expression is not regulated by these pathways. In contrast, *Fgf17* is expressed in the septum and at low levels in the roof plate ventral to the cortical hem of control embryos (Fig. 2A-C, G-I) but shows ectopic expression in the cortical hem and adjacent hippocampal primordium of all three *Gli3* mutants while its septal expression is not affected (Fig. 2D-F, J-L) suggesting that *Fgf17* expression in the dorsomedial telencephalon is repressed in a *Gli3* dependent manner likely through Bmp and/or Wnt/ β -catenin signalling. Finally, *Fgf15* has the widest *Fgf* expression domain encompassing the septum and the interganglionic sulcus while the cortical primordium is devoid of *Fgf15* transcripts (Fig. 2M-O, S-U). In contrast, all *Gli3* mutants show a dramatic up-regulation of *Fgf15* expression in cortical progenitors (Fig. 2P-R, V-X) strongly suggesting that its cortical expression is repressed by *Gli3*. Taken together, these findings suggest that the partially overlapping but distinct expression patterns of *Fgfs* in the telencephalon are generated by differential regulation through *Gli3*, *Bmps* and/or *Wnts* signalling.

Activity of an *Fgf17* forebrain enhancer is repressed by Wnt/ β -catenin signalling

Next, we investigated the molecular basis for this differential regulation by analysing regulatory elements directing *Fgf* gene expression in the forebrain. *Fgf8* expression is controlled by multiple and distinct regulatory modules that act in a holo-enhancer as a coherent unit (Vesely J *et al.* 1994) and this control can only be studied within the context of the holo-enhancer. The regulation of *Fgf18* is less well characterised, there is currently no regulatory element available directing *Fgf18* expression.

A 3.5 kb region immediately upstream of the *Fgf15* promoter was shown to direct *lacZ* reporter gene expression in the diencephalon and midbrain of E9.5 transgenic mouse embryos and to contain a Gli binding site essential for its activity (Pinto L *et al.* 2009; Mi D *et al.* 2013). However, our in utero electroporation showed that this element is not active in the E13.5 dorsomedial and

dorsolateral telencephalon and mutations in the Gli binding site did not result in enhancer activity in the dorsal telencephalon either (Sup. Fig. 2). Thus, *Fgf15* expression in the telencephalon is regulated by currently unknown regulatory elements located outside this 3.6 kb region.

Finally, two evolutionarily conserved *Fgf17* regions were shown to control *lacZ* reporter expression in the forebrain (Visel A et al. 2008). An intragenic *Fgf17* enhancer (Vista Enhancer element # 782) shows specific activity in the septum while an enhancer (Vista Enhancer element # 781) located in the intergenic region between *Fgf17* and *Epb4.9*, which is not expressed in the telencephalon, is active in the telencephalic roof plate as well as in the septum (Sup. Fig. 3). Since these two enhancers accurately reflect expression of the endogenous *Fgf17* gene, they were used for further analysis. Sequence inspection revealed a single evolutionarily conserved Tcf/Lef binding site in both enhancers, however, a recombinant full-length Lef1 protein fused to glutathione-S-transferase (GST-Lef1) only bound to the intergenic *Fgf17* enhancer but not to the *Fgf17* intragenic regulatory element in electromobility shift assays (Sup. Fig. 4). Binding to the oligonucleotide from the intragenic enhancer was specific as it was competed by unlabelled wild-type oligonucleotide but not by one containing two point mutations which abolish Lef1 binding (Hasenpusch-Theil K et al. 2012) (Fig. 3A). We next tested the functionality of this Tcf/Lef binding site within the *Fgf17* intragenic enhancer by electroporating a wild-type *Fgf17/lacZ* (*Fgf17/lacZ*) and a Tcf mutant *Fgf17/lacZ* (*mTcfFgf17/lacZ*) reporter gene construct into the telencephalon. Electroporations of the *Fgf17/lacZ* construct resulted in X-Gal staining in the septum (n=5/6 embryos) but not in the cortex (n=0/4 embryos) or in the hippocampal primordium (n=0/6 embryos) (Fig. 3B-E) reflecting the expression of the endogenous *Fgf17* gene. In the rostral telencephalon, the *mTcfFgf17/lacZ* construct showed an identical activity pattern with lacZ staining in the septum (n=5/5 embryos) but not in the cortex (n=0/3 embryos) (Fig. 3F, G). In contrast, the mutant construct was ectopically active in the cortical hem and in the hippocampus (n=5/6 embryos) (Fig. 3H, I) where *Fgf17* is ectopically expressed in *Gli3* mutant but not in wild-type embryos. These findings suggest that Wnt/ β -catenin signalling represses the activity of the intergenic *Fgf17* enhancer in the cortical hem and hippocampus. To obtain further evidence for the ability of Wnt/ β -catenin signalling to regulate *Fgf17* expression, we determined the consequences of ectopic activation of this pathway on endogenous *Fgf17* expression. To this end, we employed an ex vivo explant assay (Hasenpusch-Theil K et al. 2012) in

which we prepared coronal sections of the E13.5 mouse telencephalon and maintained these sections in culture for 24h in the presence of DMSO or various concentrations of CHIR99021 (CHIR) which selectively inhibits GSK3 β and thereby activates Wnt/ β -catenin signalling (Ring DB et al. 2003). Under control conditions, *Fgf17* expression is confined to the commissural plate (Fig. 3J). This expression pattern is maintained in the presence of 25 μ m CHIR (Fig. 3K) but treatment with 50 μ m CHIR led to a severe reduction of *Fgf17* expression (Fig. 3L). These findings indicate that *Fgf17* expression can be repressed by Wnt/ β -catenin signalling.

Fgf and Wnt/ β -catenin signalling cooperate to control the activity of a *Gli3* forebrain enhancer in the dorsomedial telencephalon and in the thalamus

Finally, we analyzed the transcriptional regulation of *Gli3*. Our previous analyses showed that the activity of a *Gli3* forebrain enhancer is directly regulated by Wnt/ β -catenin signalling (Hasenpusch-Theil K et al. 2012). Close examination of the enhancer sequences also identified four evolutionarily conserved ^A/_GAGGAA^A/_G^G/_A motifs in the *Gli3* forebrain enhancer raising the possibility that Ets transcription factors and hence Fgf signalling could regulate *Gli3* enhancer activity. Oligonucleotides containing the predicted Ets binding motifs were therefore tested for binding to GST-Etv4 in EMSAs. In this assay, GST-Etv4 specifically bound to all four Ets binding sites showing, however, different affinities with sites #1 and #4 showing the strongest and site #3 the weakest binding (Fig. 4). We next tested whether these sites are essential for *Gli3* forebrain enhancer activity by generating transgenic reporter gene embryos containing point mutations in these sites to abolish Ets binding. The wild-type *Gli3* enhancer directed strong reporter gene expression in E11.5 telencephalon and in the thalamus as described previously (Paparidis Z et al. 2007; Visel A et al. 2008; Hasenpusch-Theil K et al. 2012) (Fig. 5A). In contrast, a construct containing mutations in all four Ets binding sites led to reduced and patchy *lacZ* expression in the dorsomedial telencephalon and to a complete loss of enhancer activity in the thalamus whereas expression in the dorsolateral telencephalon is not affected (n=5/9 transgenic embryos) (Fig. 5B). To analyse the individual

contributions of these sites, we tested *Gli3* enhancer activity after introducing mutations in combinations of the Ets binding sites. Intact binding sites #3 and #4 were not sufficient to restore enhancer activity as constructs containing intact Ets binding sites #3 or #3+4 but mutations in sites #1 and #2 still showed loss of enhancer activity in the dorsomedial telencephalon and in the thalamus (n=5/9 transgenic embryos for each construct) (Fig. 5C, D) suggesting that binding sites #1 and #2 are essential for this enhancer activity. We therefore tested the contribution of binding site #1 and #2 by individually mutating these two sites. In contrast to a binding site #1 mutant construct which produced the wild-type *lacZ* expression pattern (n=2/4 transgenic embryos) (Fig. 5E), embryos transgenic for the binding site #2 mutant construct showed a loss of enhancer activity in dorsomedial telencephalon and in the thalamus (n=5/10 transgenic embryos) (Fig. 5F). In addition, we noted that embryos transgenic for constructs lacking site #2 but with an intact site #3 have a broader loss of enhancer activity in the dorsomedial telencephalon (Fig. 5C, D, F) suggesting that site #3 might repress the activity of site #2. Thus, Ets binding site #2 is crucial for *Gli3* forebrain enhancer activity in these tissues whereas binding site #3 might have a modulatory effect on the activity of site #2.

Interestingly, Ets binding site #2 lies immediately adjacent to the essential Tcf/Lef binding site we previously identified (Hasenpusch-Theil K *et al.* 2012) raising the possibility that Ets and Tcf/Lef transcription factors might cooperate in regulating the *Gli3* forebrain enhancer. To test this possibility, we performed EMSA analyses with an oligonucleotide containing the Tcf/Lef and Ets #2 binding sites and recombinant GST-Lef1 and/or GST-Etv4 fusion protein. Incubation with the single proteins led to the formation of a single complex (Fig. 6). The formation of the GST-Lef1 complex, however, was increased in the presence of both proteins (Fig. 6) suggesting that Etv4 increases the binding affinity for Lef1 to its binding site within the *Gli3* forebrain enhancer.

DISCUSSION

The *Gli3* mediated balance between *Fgfs* and *Wnts* is of pivotal importance for the control of telencephalic patterning and axon tract formation but very little is known about the molecular basis by which this balance is established and/or maintained. Here, we investigated direct interactions between *Gli3* and these signalling pathways at the transcriptional level. We show that cortical hem activity of the *Wnt8b* dorsomedial telencephalon enhancer is positively regulated by Fgf signalling, while in turn *Fgf17* expression is directly repressed by Wnt/ β -catenin signalling. In addition, both signalling pathways cooperate in directly regulating *Gli3* forebrain enhancer activity in the dorsomedial telencephalon. Taken together, these interactions form the basis of a key element regulating telencephalic patterning.

Differential expression of *Fgfs* in the telencephalon coincides with differences in regulation and functions

Multiple *Fgfs* with unique or redundant functions are involved in telencephalic patterning. Among the *Fgf8/Fgf17/Fgf18* subfamily, *Fgf8* is the key factor in telencephalic patterning (Louvi A and EA Grove 2011; van den Ameele J *et al.* 2014) while *Fgf17* has a more limited role in controlling the patterning of the rostral cortical primordium (Cholfin JA and JL Rubenstein 2007; Petrova R *et al.* 2013). A role for *Fgf18* in the rostral patterning centre has not been established yet but it has been implicated in controlling the migration of cortical neurons (Finn RS *et al.* 2009). In contrast, *Fgf15* opposes the function of *Fgf8* (Han YG *et al.* 2008). This functional diversity is at least partially reflected in their complex and partially overlapping expression patterns but little is known how this differential expression is established and maintained. Our analyses of *Fgf* gene expression in *Gli3* mutants provided first insights into this regulation. While *Fgf18* expression is not affected, *Fgf8* and *Fgf17* show a specific up-regulation in dorsal midline tissues in the *Gli3* mutants. In contrast, *Fgf15* is ectopically expressed throughout the cortex of these mutants. These different effects of *Gli3* mutations on *Fgf* gene expression suggest differential modes of transcriptional regulation. Due to their confined up-regulation, *Fgf8* and *Fgf17* could be regulated by Bmp and/or Wnt/ β -catenin

signalling. Indeed, using bandshift and reporter gene analyses we show that the activity of the intergenic *Fgf17* forebrain enhancer is repressed in the dorsomedial telencephalon by Wnt/ β -catenin signalling. In addition, pharmacological activation of Wnt/ β -catenin signalling in an ex vivo explant assay resulted in a severe reduction of *Fgf17* expression in the commissural plate. Taken together with the ectopic *Fgf17* expression in the *Gli3* mutant dorsomedial telencephalon this finding indicates that *Fgf17* represents one of the few known directly repressed target genes for Wnt/ β -catenin signalling. Interestingly, a second *Fgf17* forebrain enhancer shows an overlapping activity in the septum but is not active in the telencephalic roof plate consistent with the failure of GST-Lef1 protein to bind to a potential Tcf/Lef binding site within this enhancer in our bandshift assay. While this lack of binding is likely to be explained by negative influences of neighbouring sequences, the differential response of both enhancers to Wnt/ β -catenin signalling suggests that the existence of both enhancers allowed the development of different regulatory output while maintaining a core activity in the septum as has been reported for other shadow enhancers (Paridaen JT and WB Huttner 2014; Magnani D et al. 2015; Paridaen JT et al. 2015). Regulation of *Fgf8* expression in the forebrain appears to be even more complex. Recently, an extensive analysis of *Fgf8* expression during embryogenesis revealed the existence of multiple enhancers distributed over a 200kb stretch forming the *Fgf8* holo-enhancer (Vesely J et al. 1994). In an identical enhancer assay to that used in our analysis, a couple of these elements (CE63 and CE64) display widespread activity in the telencephalon which only becomes confined to the commissural plate by filtering through the holo-enhancer. Interestingly, our sequence analysis revealed several potential Tcf/Lef binding sites in these two elements suggesting that Wnt/ β -catenin signalling might be involved in regulating their activity and hence *Fgf8* expression, but this hypothesis needs to be tested within the context of the holo-enhancer.

Fgf15 dramatically differs from other telencephalic Fgfs both in functional as well as in regulatory terms. Its activity as an *Fgf8* antagonist coincides with a widespread up-regulation throughout the dorsal telencephalon of the *Gli3* mutants analysed here and in *Gli3*^{Xt/Xt} mutants (Rash BG and EA Grove 2007). This ectopic expression taken together with *Fgf15* expression in the interganglionic sulcus where Shh signalling is active raised the possibility that *Gli3* directly represses *Fgf15*

expression in the dorsal telencephalon. However, a mutation in a Gli binding site which has previously been shown to be critical for the activity of an *Fgf15* ventral midbrain/diencephalon enhancer did not result in reporter gene expression in the dorsal telencephalon. This finding suggests that different, currently unknown regulatory elements outside the promoter region may be responsible for this repression or that this repression is indirect. Regardless of the exact mechanism, the repression of *Fgf15* expression by *Gli3* is likely to be of functional importance since ectopic *Fgf15* expression in the E14.5 dorsal telencephalon causes premature differentiation of cortical progenitors (Paridaen JT et al. 2013) but may also limit the effects of ectopic *Fgf8* expression in *Gli3* mutants due to its antagonistic effects on *Fgf8* (Han YG et al. 2008).

Fgf signalling regulates the activity levels of the *Wnt8b* dorsomedial enhancer

In addition to the direct repression of *Fgf17* by Wnt/ β -catenin signalling, we provide evidence that Fgf signalling in turn directly regulates *Wnt8b* expression in the cortical hem. The *Wnt8b* dorsomedial telencephalon enhancer contains an *Ets* binding site mutation of which results in loss of enhancer activity in the hem. Moreover, ectopic expression of activator and repressor forms of the *Etv4* transcription factors led to activation and repression of the *Wnt8b* enhancer in the rostromedial telencephalon, respectively. Taken together, these findings strongly suggest that *Wnt8b* is a direct target for Fgf signalling. Previously, it was shown that *Wnt8b* expression is repressed by *Foxg1* via an evolutionarily conserved binding site in the *Wnt8b* promoter (Wilsch-Brauninger M et al. 2012) distinct from the *Wnt8b* dorsomedial telencephalon enhancer. Hence, *Fgfs* can regulate *Wnt8b* expression by two independent mechanisms, either directly via the *Ets* binding site in the dorsomedial telencephalon enhancer or indirectly through *Foxg1*.

The mutual interactions between *Fgfs* and *Wnts* form an important aspect of patterning. The rostral telencephalon is peculiar in that domains of *Fgf15/17/18* and *Wnt7b/Wnt8b* expression are juxtaposed (Higginbotham H et al. 2013). This juxtaposition combined with the mutual interactions provides a mechanism by which not only stable expression domains of these factors are generated but which also limit the extent of Fgf and Wnt/ β -catenin signalling. This mechanism appears of particular importance in light of the *Wnt8b* auto-regulation (Hasenpusch-Theil K et al. 2012).

Fgf and Wnt/ β -catenin signalling cooperate in regulating *Gli3* expression in the dorsomedial telencephalon

In addition to the mutual interactions between Fgf and Wnt/ β -catenin signalling, both pathways cooperate in regulating the activity of the *Gli3* forebrain enhancer. Previously, we showed that the activity of this enhancer depends on a Tcf/Lef binding site (Hasenpusch-Theil K *et al.* 2012). Here, we extend this finding by showing Fgf signalling is also involved in regulating the activity of the *Gli3* forebrain enhancer. While Ets binding site #3 might have a modulatory effect, Ets binding site #2 immediately adjacent to the Tcf binding site is required for enhancer activity specifically in the dorsomedial telencephalon and in the dorsal diencephalon. This spatially confined effect coincides with the known expression of *Fgfs* and with the activity of Fgf signalling in both tissues (Zimmer C *et al.* 2010). Moreover, the presence of Ets protein increases the binding of Lef1 protein to an oligonucleotide from the *Gli3* enhancer in a bandshift assay. Interestingly, this increased binding does not result from the simultaneous binding of both transcription factors suggesting that Ets4 might increase Lef1 binding activity in a DNA independent manner. Taken together, these findings strongly suggest that both signalling pathways converge to regulate *Gli3* expression in the dorsomedial telencephalon and dorsal diencephalon.

This cooperative interaction in the regulation of *Gli3* expression combined with the mutual interactions between Fgf and Wnt/ β -catenin signalling confer stability and robustness to the telencephalic patterning process. Based on genetic analyses, a model was proposed that unidentified factors promoting cortical specification in the dorsal telencephalon are controlled by Fgf activity which in turn is disinhibited by Gli3 repressor mediated inhibition of *Fgf* gene expression (Rash BG and EA Grove 2007). Our analyses extend this model and clarify the molecular basis of some of these interactions (Fig. 7). Due to their prominent role in dorsal telencephalic development (Machon O *et al.* 2007; Mangale VS *et al.* 2008) and due to their direct control of *Gli3* expression (Hasenpusch-Theil K *et al.* 2012), *Wnts* are likely to be at least one of the factors specifying cortical cell fates and directing early cortical differentiation. Direct regulation of *Wnt8b* expression in the hem by Fgf signalling and a mutual and direct repression of *Fgf17* and possibly *Fgf8* expression by Wnt/ β -

catenin signalling provide a balance between these two pathways. Moreover, *Gli3* connects these two pathways with the third major signalling pathway, Shh signalling, and hence is a central factor in cortical patterning by suppressing ventral telencephalic cell fates and by promoting specification of dorsal telencephalic cell fates through its control of the expression of multiple Fgfs and Wnts. Thus, the interconnectedness between *Gli3*, *Wnt* and *Fgf* genes takes centre stage in conferring robustness to the telencephalic patterning process. This tight control is essential to cortical development since subtle changes in *Fgf* or *Gli3* expression levels as in *Fgf8* hypomorphic and *Gli3*^{Pdn/Pdn} mutants, respectively, or in the Gli3 activator/Gli3 repressor ratio as in ciliary mouse mutants can have profound effects not only on telencephalic patterning (Willaredt MA et al. 2008) but also on subsequent developmental processes. For example, small differences in *Gli3* expression levels or in Gli3 processing have been hypothesized to form the basis for agenesis of the corpus callosum in *GLI3* and ciliopathy patients (Vokes SA et al. 2008; Speksnijder L et al. 2013; Magnani D et al. 2014; Laclef C et al. 2015). Moreover, gradients of Fgf and Wnt signalling molecules are crucial determinants of cortical arealization and, based on the protomap hypothesis (Rakic P 2009; Guo J et al. 2015), changes in the levels of *Fgf* and *Wnt* gene expression have been suggested to play an important role in the evolution of cortical areas. Our study provides a framework for understanding how these gradients are set up early in cortical development during regionalization of the dorsal telencephalon.

ACKNOWLEDGEMENTS

We thank John Mason and David Price for critical comments on the manuscript and Bob Hill for invaluable advice on EMSAs. We are grateful to Andrew Sharrocks for providing the GST-Etv4 expression plasmid. We are particularly indebted to Emma Allan at the Western General Hospital for the expert generation of transient transgenic mouse embryos. This work was supported by a grant from the Medical Research Council (MR/K013750/1).

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FIGURE LEGENDS

Figure 1: An Ets binding site regulates activity levels of the *Wnt8b* dorsomedial telencephalon enhancer. (A) Electromobility shift assays showing in vitro binding of recombinant Etv4 protein to the *Wnt8b* enhancer (lane 2). Complex formation is competed by increasing amounts of wild-type enhancer oligonucleotide (lane 3 and 4) but not by oligonucleotides containing point mutations in the Ets binding site (lanes 5 and 6). (B, C) The *Wnt8b* enhancer shows strong activity in the roof plate (rp), in the dorsomedial telencephalon (t) including the cortical hem and in preplate neurons in the dorsolateral telencephalon of transgenic embryos. The arrow in (C) demarcates the ventral most expression in preplate neurons (G, H). Mutation in the *Ets* binding site led to loss of enhancer activity in the cortical hem. Note the ventrally expanded expression domain in preplate neurons (H). The number of transgenic embryos is shown at the bottom of the figures. (D, I) Electroporation of the *Wnt8b/lacZ* reporter results in enhancer activity in the rostral midline region. (E, F, J, K) Co-electroporation of an Etv4-E1A expression construct resulted in a dorsal expansion of lacZ staining (J) while an Etv4-EngR construct reduced lacZ staining (K). The arrows in (I-L) indicate the dorsal most extent of the *lacZ* expression domain. (D-F) GFP immunofluorescence revealed the electroporation sites. Abbreviations: ctx, cortex; MGE, medial ganglionic eminence; LGE, lateral ganglionic eminence; sep, septum. Scale bars: (B, G): 1mm; (C-F, H-K) 100µm.

Figure 2: *Fgf15* and *17* expression in the *Gli3* mutant telencephalon. *Fgf17* (A-L) and *Fgf15* (M-X) in situ hybridization on E12.5 control (A-C, G-I, M-O, S-U), *Gli3*^{Pdn/Pdn} (D, J, P, V), *Gli3*^{Xt/Pdn} (E, K, Q, W) and *Emx1Cre;Gli3*^{fl/fl} (F, L, R, X) embryos. (A-C, G-I) *Fgf17* expression is restricted to the septum (sep) rostrally and to the roof plate (rp) caudally. (D-F, J-L) *Fgf17* expression in the septum is not affected in *Gli3* mutants (D-F) while transcripts were ectopically detected in the ventral most hippocampal primordium (hp). (M-O, S-U) *Fgf15* expression is restricted to the septum and to the interganglionic sulcus between medial and lateral ganglionic eminence (MGE and LGE). (P-R, V-X) In *Gli3* mutants, *Fgf15* is ectopically expressed in the cortex (ctx). Scale bars: 100µm.

Figure 3: A Tcf/Lef binding site represses *Fgf17* enhancer activity in the dorsomedial telencephalon. (A) Electromobility shift assays showing in vitro binding of recombinant GST-Lef1 protein to the *Fgf17* enhancer (lane 3). Complex formation is competed by increasing amounts of wild-type enhancer oligonucleotide (lane 4, 5 and 6) but not by oligonucleotides containing point mutations in the Tcf/Lef binding site (lanes 7, 8 and 9). (B-E) Electroporation of a *GFP* expression construct and an *Fgf17/lacZ* reporter plasmid into the rostromedial telencephalon leads to enhancer activity in the septum (arrows in C) but not in the rostral cortex (C) or in the hippocampal (h) primordium (E). (F-I) A *Tcf/Lef* mutant *Fgf17/lacZ* construct shows enhancer activity in the septum (arrows in G) and ectopic lacZ staining in the cortical hem and in the hippocampus (arrows in I). (J-L) Ex vivo explant assay to determine the effects of ectopic Wnt/β-catenin signalling on *Fgf17*

expression. (J, K) *Fgf17* expression in the commissural plate (cp) after DMSO (J) and 25 μ M CHIR treatment (K). (L) In the presence of 50 μ M CHIR, *Fgf17* expression is severely reduced. Scale bars: (B-E, F-I): 100 μ m; (J-L): 200 μ m.

Figure 4: Electromobility shift assays showing in vitro binding of recombinant Etv4 protein to the *Gli3* forebrain enhancer. The schematic indicates the position of the four Etv4 and the Tcf/Lef binding sites in the *Gli3* forebrain enhancer. GST-Etv4 protein binds to oligonucleotides of the *Gli3* forebrain enhancers (lane 2) though with different affinities. In each case, complex formation is progressively competed by increasing amounts of wild-type enhancer oligonucleotide (lanes 3 and 4) but not by oligonucleotides containing point mutations in the Ets binding site (lanes 5 and 6).

Figure 5: Ets binding site 2 is essential for *Gli3* enhancer activity in the dorsomedial telencephalon and in the thalamus. (A) The *Gli3* forebrain enhancer is active in the dorsomedial telencephalon (t) and in the dorsal diencephalon (d). (B) Mutations of all four Ets binding sites lead to reduced *lacZ* expression in the dorsomedial telencephalon and abolish enhancer activity in the thalamus. (C, D) Embryos transgenic for the mutant Ets₁₊₂₊₄ (C) and Ets₁₊₂ (D) constructs lack enhancer activity in the dorsomedial telencephalon and in the diencephalon. (E, F) Mutation of Ets binding site 1 has no effect on enhancer activity (E) while embryos transgenic for the *Gli3* reporter construct carrying a mutation in Ets binding site 2 show no *lacZ* staining in the dorsomedial telencephalon and in the thalamus (F). Scale bars: 100 μ m.

Figure 6: The presence of Etv4 protein enhances the binding of Lef1 protein to the *Gli3* forebrain enhancer. The sequence indicates the relative position of the Tcf/Lef and Ets binding sites. GST-Etv4 and GST-Lef1 bind individually to an oligonucleotide from the *Gli3* enhancer containing both binding sites while the binding of GST-Lef1 is increased in the presence of GST-Etv4 protein.

Figure 7: Model describing interactions between *Gli3*, *Shh*, *Wnt8b* and several *Fgfs* underlying cortical development. Specification of the cortex involves the repression of *Shh* signalling via *Gli3* and Wnt/ β -catenin signalling. *Gli3* is also required for establishing *Wnt8b* expression through an unknown mechanism. *Wnt8b* expression is directly activated by Fgf signalling and maintains its own expression through an autoregulatory mechanism. In turn, Wnt/ β -catenin signalling directly represses *Fgf17* expression. Finally, Fgf and Wnt/ β -catenin signalling cooperate in regulating *Gli3* expression.

FIGURES

Figure 1

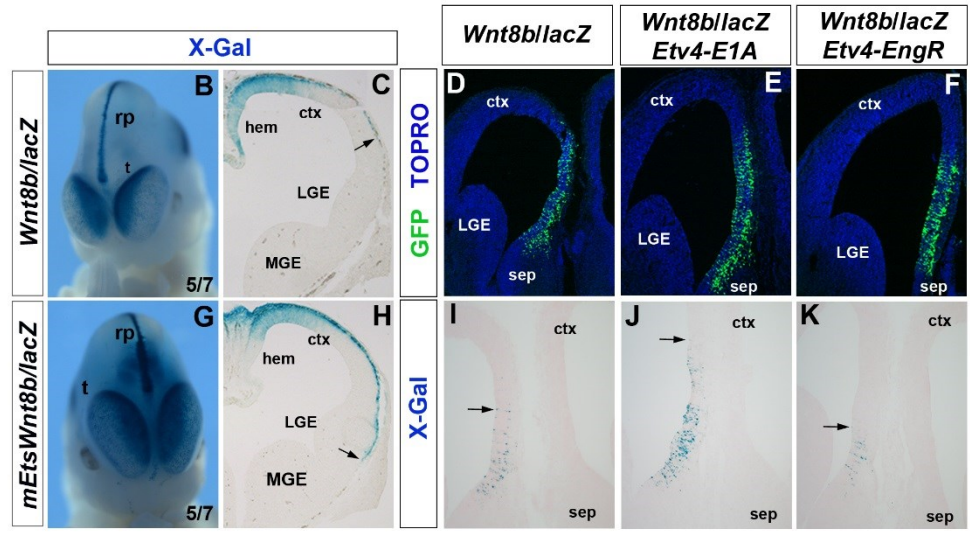
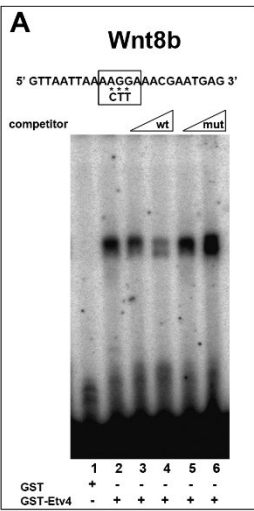


Figure 2

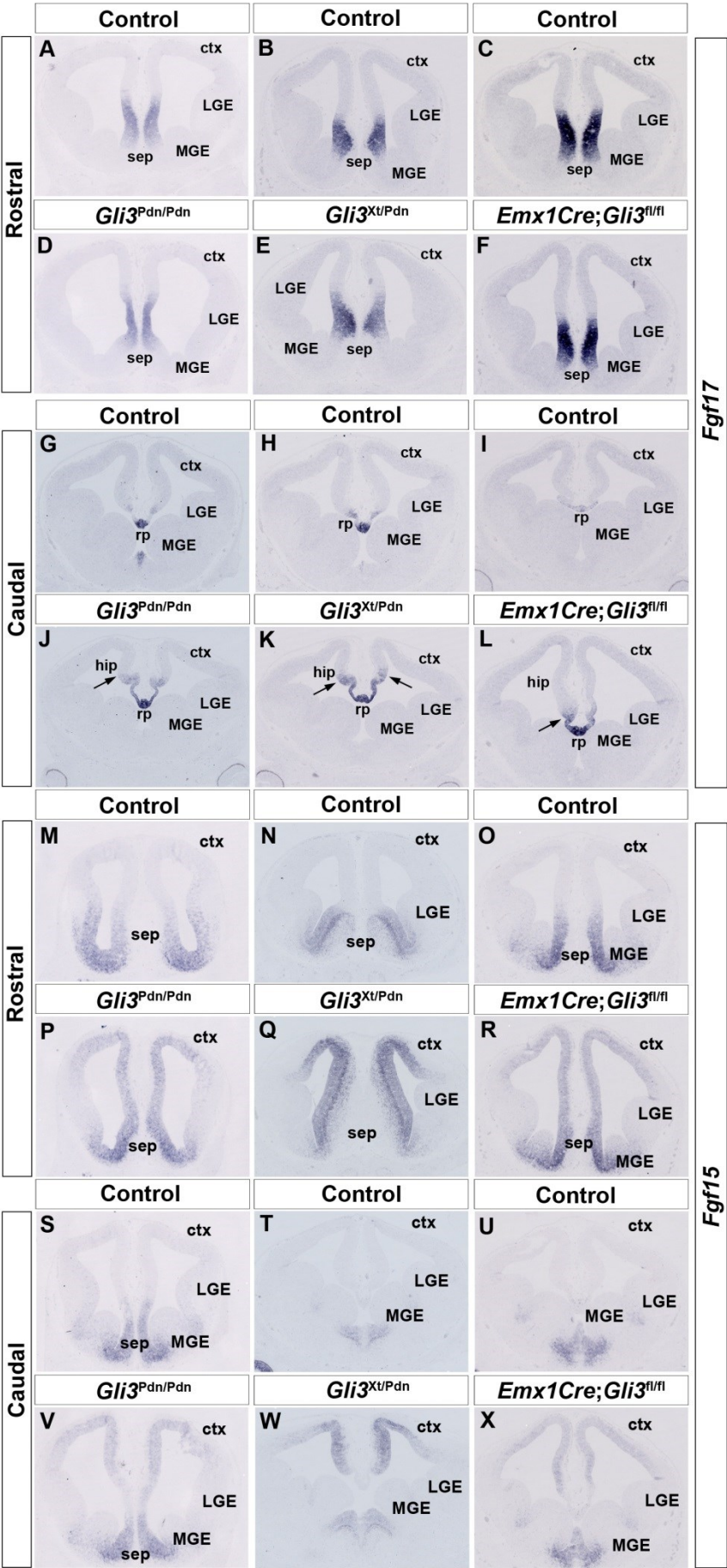


Figure 3

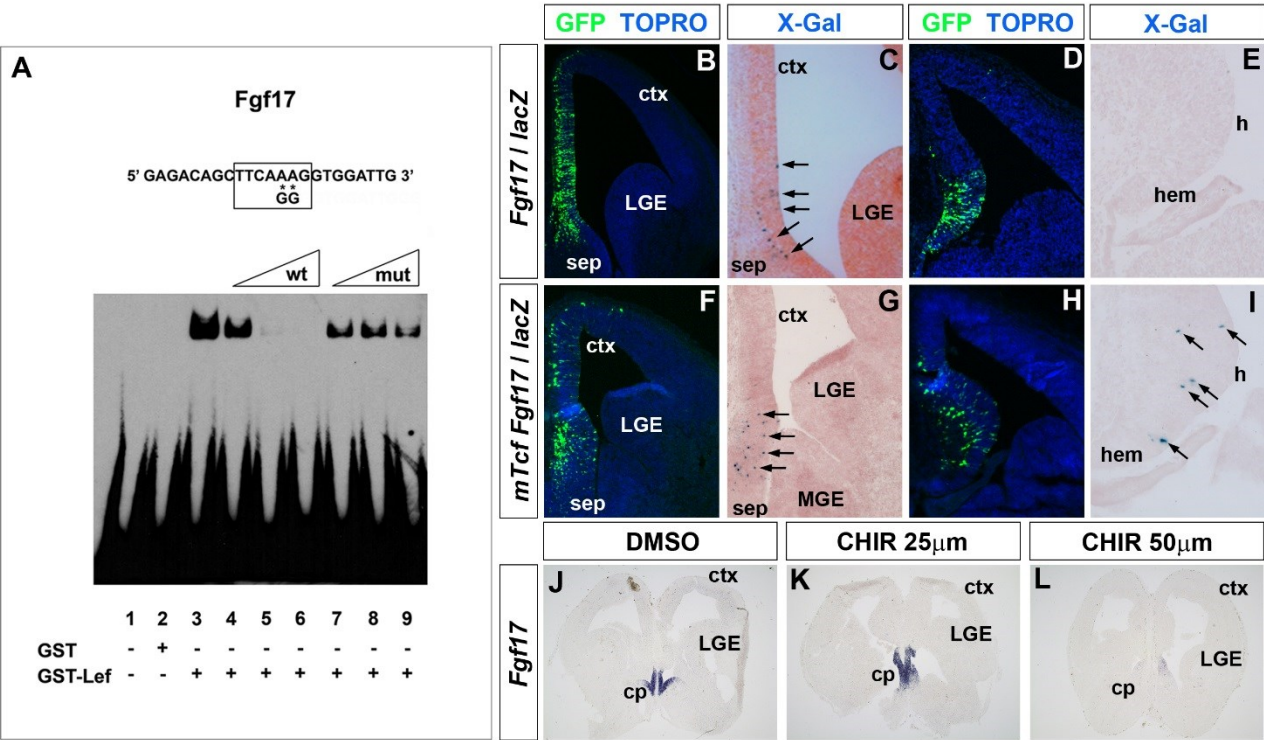


Figure 4

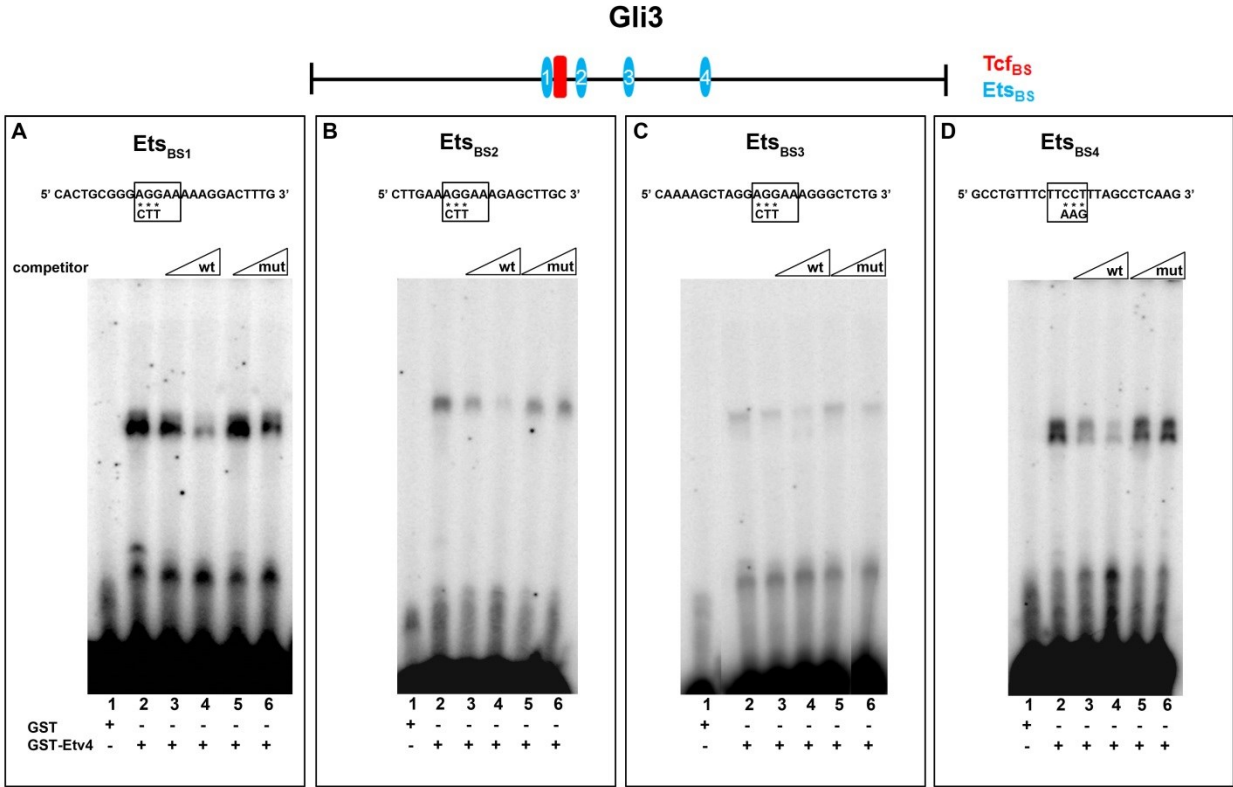


Figure 5

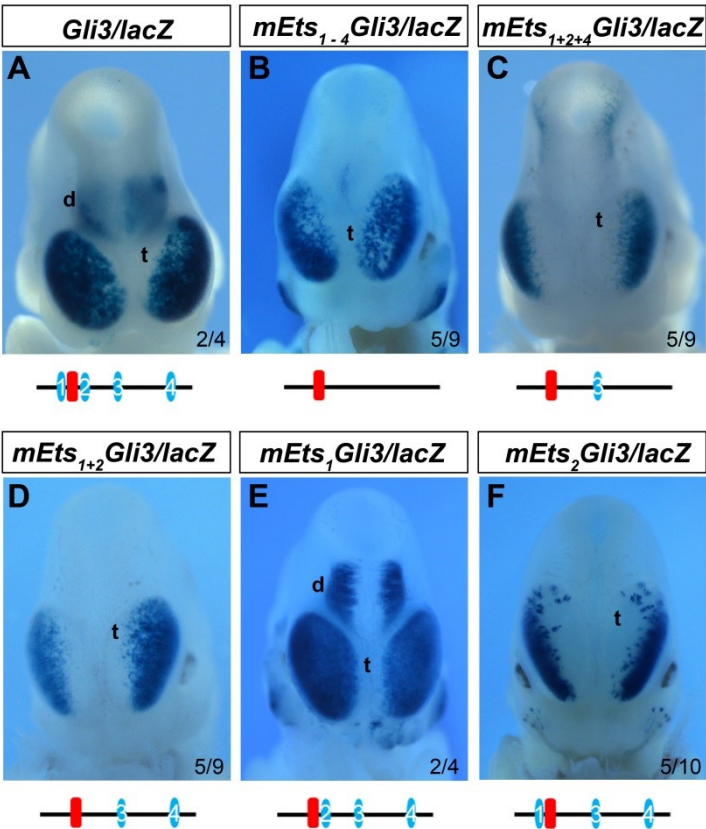


Figure 6

5' AGGACTTTGAAACTTGAAAGGAAAGAGCTTGC 3'

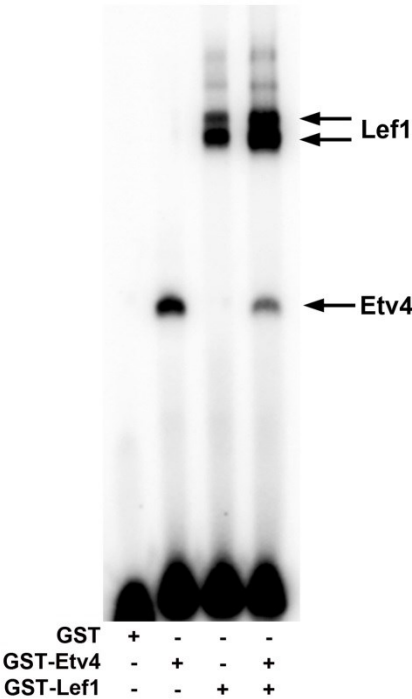
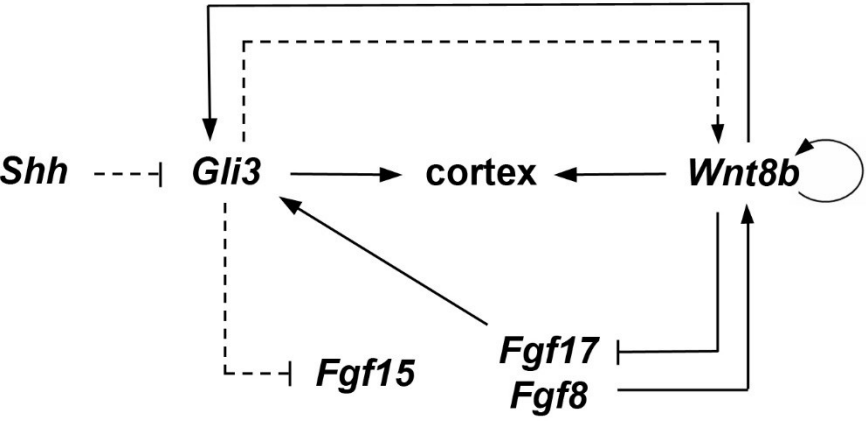
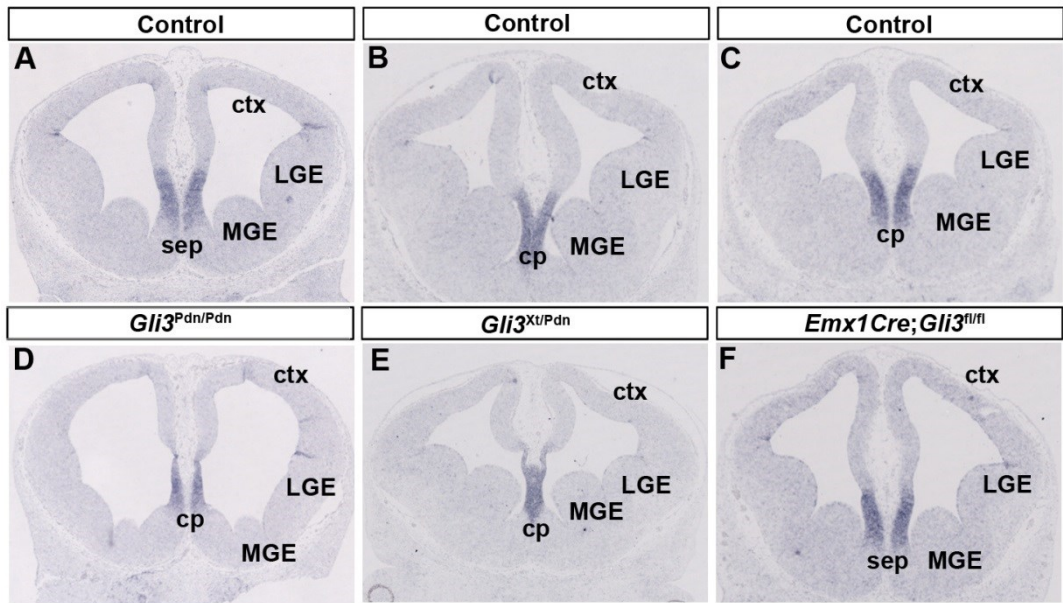


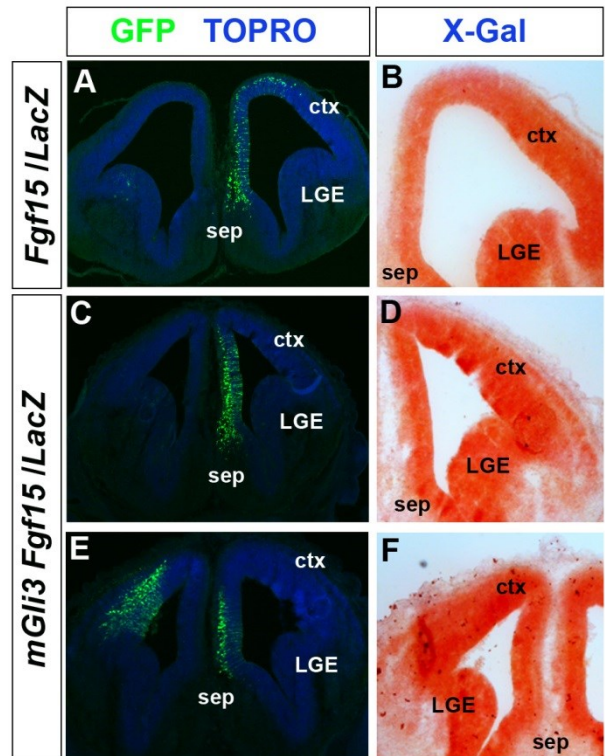
Figure 7



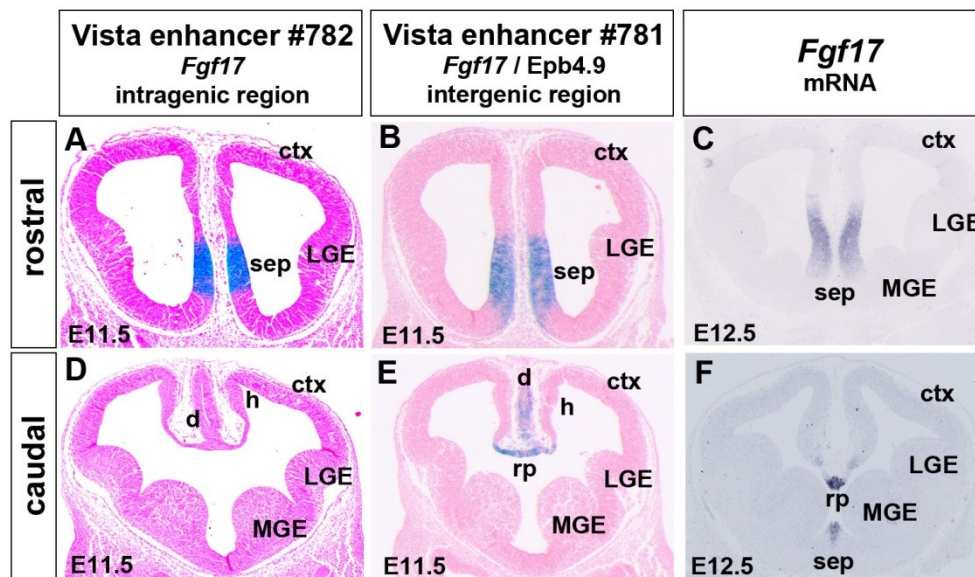
SUPPLEMENTARY FIGURES



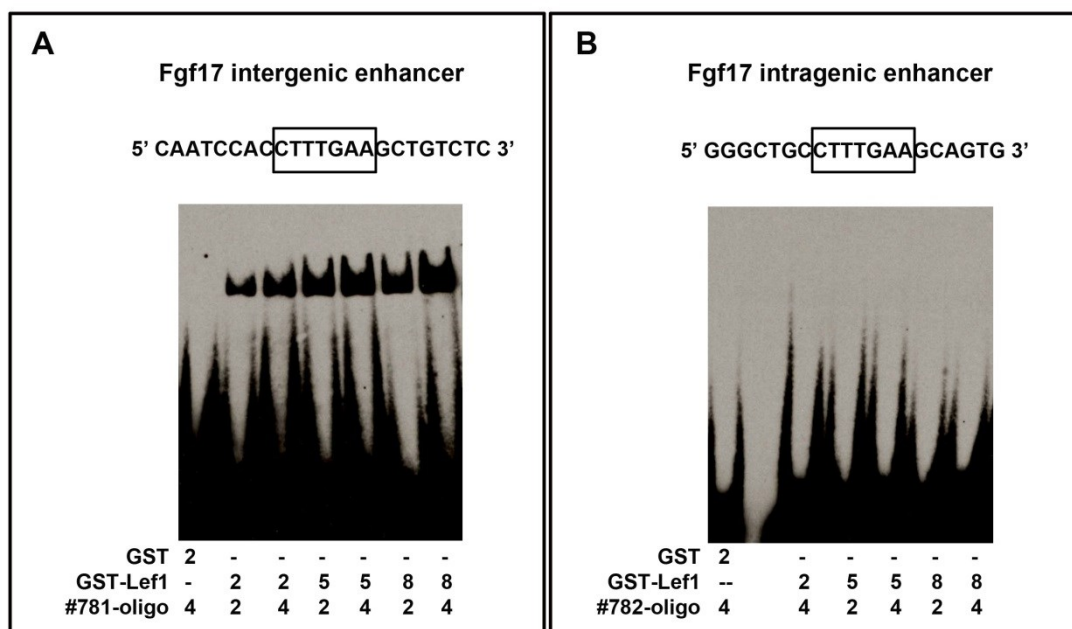
Supplementary Figure 1: *Fgf18* expression in the *Gli3* mutant telencephalon. *Fgf18* in situ hybridization on E12.5 control (A-C), *Gli3*^{Pdn/Pdn} (D), *Gli3*^{Xt/Pdn} (E) and *Emx1Cre;Gli3*^{fl/fl} (F) embryos. *Fgf18* expression is restricted to the commissural plate of control embryos and is not obviously affected by the *Gli3* mutations.



Supplementary Figure 2: The activity of the *Fgf15* promoter is not repressed by *Gli3* in the dorsal telencephalon. Co-electroporation of GFP with either a wild-type *Fgf15*/lacZ reporter plasmid (A, B) or with a reporter plasmid carrying a mutation in a *Gli3* binding site (C-F) does not lead to enhancer activity in the dorsal telencephalon.



Supplementary Figure 3: *Fgf17* forebrain enhancers. Comparison of *Fgf17* forebrain enhancer activity with *Fgf17* mRNA expression. (A, D) Vista enhancer #782 located in the *Fgf17* intragenic region is active in the septum but not in the roof plate. (B, E) Activity of the Vista enhancer #781 situated in the *Fgf17*/*Epb4.9* intergenic region is found in the septum and at more caudal levels in the roof plate and in the diencephalon (d) reflecting the expression pattern of the endogenous *Fgf17* gene (C, F).



Supplementary Figure 4: GST-Lef1 fusion protein binds to the *Fgf17* intergenic enhancer but not to the *Fgf17* intragenic enhancer. EMSA analysis using oligonucleotides from the *Fgf17* intergenic (A) and *Fgf17* intragenic enhancers (B) and increasing amounts of GST-Lef1 fusion protein. Only the oligonucleotide from the *Fgf17* intragenic enhancer binds Lef1 protein.

Supplementary Table 1

Vista enhancer ID	fragment length (bp)	gene	primer	primer sequence (5' -3')
hs781	724	Fgf17	781_F1	GAGCTGGGACGGAGCCTGACA
			781_R1	CTGCTCTCCCAAGCTGCTCTTC

GenBank ID	fragment length (bp)	gene	primer	primer sequence (5' -3')	restriction sites used for cloning
NC_000073.6	3501	Fgf15	Fgf15_Fwd1	AAAATTCGAACACGGCTCGC	2.2kb HindIII / SphI
			Fgf15_Rev1	CGGAGCTCTGGGAGAATGTC	
			Fgf15_Fwd2	GAAGTAAACCCGCCCTGGA	1.3kb SphI / XhoI
			Fgf15_Rev2	CCGGGCATCAGAGCATTCT	

Supplementary Table 2

Oligonucleotides used for mutagenesis

Vista enhancer ID	gene		primer	primer sequence (5' -3')	mutations introduced
	Fgf15		Fgf15_GliBS _{mtg} _Fwd	CCCCTGCAGACAGAC <u>GTGGG</u> ATCACCACGGAGCTA	(CTCCC/ <u>GTGGG</u>)
			Fgf15_GliBS _{mtg} _Rev	TAGCTCCGTGGTGAT <u>CCCAC</u> GTCTGTCTGCAGGGG	
hs781	Fgf17		781_Tcf _{mtg} _Fwd	CTGGAGACAGCTTCA <u>GGGG</u> TGGATTGGG	(AA/ <u>GG</u>)
			781_Tcf _{mtg} _Rev	CCCAATCCACC <u>CCT</u> GAAAGCTGTCTCCAG	
hs111	Gli3	Ets _{BS1mut}	G3_Ets_B1 _{mtg} _Fwd	GTGACACTGCGGG <u>CTT</u> AAAAAGGACTTTGAAAC	(AGG/ <u>CTT</u>)
			G3_Ets_B1 _{mtg} _Rev	GTTTCAAAGTCCTTTTT <u>AAG</u> CCCGCAGTGTCAC	
		Ets _{BS2mut}	G3_Ets_B2 _{mtg} _Fwd	GAAACTTGA <u>ACTT</u> AAAGAGCTTGCTTTCAAC	(CCT/ <u>AAG</u>)
			G3_Ets_B2 _{mtg} _Rev	GTTGAAAGCAAGCTCTTT <u>AAG</u> TTCAAGTTTC	
		Ets _{BS3mut}	G3_Ets_B3 _{mtg} _Fwd	CAACCTCAAAAGCT <u>AGG</u> CTTAAAGGGCTCTGAAAT	(AGG/ <u>CTT</u>)
			G3_Ets_B3 _{mtg} _Rev	ATTCAGAGCCCTTTAAG <u>CCT</u> AGCTTTTGAGGTTG	
		Ets _{BS4mut}	G3_Ets_B4 _{mtg} _Fwd	CCATTAGCCTGTTTCTT <u>AAG</u> TTAGCCTCAAG	(CCT/ <u>AAG</u>)
			G3_Ets_B4 _{mtg} _Rev	CTTGAGGCTAA <u>CTT</u> AAGAAACAGGCTAATGG	
hs1006	Wnt8b		W8b_Ets_B1 _{mtg} _Fwd	CCACTCATTGTTTT <u>AAG</u> TTTAATTAACCCAC	(CCT/ <u>AAG</u>)
			W8b_Ets_B1 _{mtg} _Rev	GTGGGTAAATTA <u>CTT</u> AAACGAATGAGTGG	

Supplementary Table 3

Oligonucleotides used for EMSA

enhancer		primer	primer sequence (5' -3')
Fgf17_781	781 Tcf _{BS}	781_Tcf_Fwd	GAGACAGCTTCAAAGGTGGATTG
		781_Tcf_Rev	CAATCCACCTTTGAAGCTGTCTC
	781 Tcf _{BSmut}	781_Tcf _{mut} _Fwd	GAGACAGCTTCA GG GGTGGATTG
		781_Tcf _{mut} _Rev	CAATCCACCC CC TGAAGCTGTCTC
Fgf17_782	782 Tcf _{BS}	782_Tcf_Fwd	GGGCTGCCTTTGAAGCAGTG
		782_Tcf_Rev	CACTGCTTCAAAGGCAGCCC
Gli3	Gli3 Ets _{BS1}	Gli_Ets_B1_Fwd	CACTGCGGGAGGAAAAAGGACTTTG
		Gli_Ets_B1_Rev	CAAAGTCCTTTTCTCCCGCAGTG
	Gli3 Ets _{BS1mut}	Gli_Ets_B1 _{mut} _Fwd	CACTGCGGG CTT AAAAAGGACTTTG
		Gli_Ets_B1 _{mut} _Rev	CAAAGTCCTTTT AAG CCCGCAGTG
	Gli3 Ets _{BS2}	Gli_Ets_B2_Fwd	CTTGAAAGGAAAGAGCTTGC
		Gli_Ets_B2_Rev	GCAAGCTCTTCCTTTCAAG
	Gli3 Ets _{BS2mut}	Gli_Ets_B2 _{mut} _Fwd	CTTGAA CTT AAAGAGCTTGC
		Gli_Ets_B2 _{mut} _Rev	GCAAGCTCTTT AAG TTCAAG
	Gli3 Ets _{BS3}	Gli_Ets_B3_Fwd	CAAAAGCTAGGAGGAAAGGGCTCTG
		Gli_Ets_B3_Rev	CAGAGCCCTTTCCTCCTAGCTTTTG
	Gli3 Ets _{BS3mut}	Gli_Ets_B3 _{mut} _Fwd	CAAAAGCTAGG CTT AAAGGGCTCTG
		Gli_Ets_B3 _{mut} _Rev	CAGAGCCCTTT AAG CCTAGCTTTTG
	Gli3 Ets _{BS4}	Gli_Ets_B4_Fwd	GCCTGTTTCTTCCTTTAGCCTCAAG
		Gli_Ets_B4_Rev	CTTGAGGCTAAAGGAAGAAACAGGC
	Gli3 Ets _{BS4mut}	Gli_Ets_B4 _{mut} _Fwd	GCCTGTTTCTT AAG TTAGCCTCAAG
		Gli_Ets_B4 _{mut} _Rev	CTTGAGGCTAA CTT AAGAAACAGGC
	Gli3 Tcf _{BS} Ets _{BS2}	G-Tcf-Ets _{BS2} _Fwd	AGGACTTTGAACTTGAAAGGAAAGAGCTTGC
		G-Tcf-Ets _{BS2} _Rev	GCAAGCTCTTCCTTTCAAGTTTCAAAGTCCT
Wnt8b	Wnt8b Ets _{BS}	W8b_Ets_BS_Fwd	CTCATTCGTTTCCTTTTAATTAAC
		W8b_Ets_BS_Rev	GTTAATTAAGGAAACGAATGAG
	Wnt8b Ets _{BSmut}	W8b_Ets_BS _{mut} _Fwd	CTCATTCGTTT AAG TTTAATTAAC
		W8b_Ets_BS _{mut} _Rev	GTTAATTAAC CTT AAACGAATGAG